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Carbon Monoxide Production from Heme Compounds by Bacteria

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Carbon monoxide formation from heme compounds by bacteria was investigated to study microbial hemoprotein catabolism with reference to heme degradation by mammalian tissues. Hemolytic and nonhemolytic bacteria were incubated aerobically and anaerobically with the following substrates: erythrocytes, hemoglobin, myoglobin, cytochrome *c*, hematin, iron hematoporphyrin, copper hematoporphyrin, protoporphyrin, and bilirubin. After 18 hr at 37 C the evolved CO was measured by gas chromatography. None of the bacteria formed CO anaerobically. Under aerobic conditions both alpha-hemolytic *Streptococcus mitis* and hemolytic *Bacillus cereus* formed CO from all of the heme compounds tested, whereas nonhemolytic *Streptococcus mitis* did not evolve CO from any of the substrates. The hemolytic bacteria did not produce CO when the iron of heme was either replaced by copper or removed, as in copper hematoporphyrin and in protoporphyrin, respectively.

Mammalian tissues (5, 20) and algae (24) generate carbon monoxide during the catabolism of heme compounds to open-chain tetrapyrroles. Tracer studies have shown that this CO is derived from the alpha methene bridge carbon of the porphyrin ring (2, 5, 10, 13, 25, 26) and molecular oxygen (23). This reaction does not proceed anaerobically (5, 13, 23). To ascertain whether bacteria can also evolve CO from hemoproteins, hemolytic and nonhemolytic strains have been incubated with heme and other porphyrin substrates under both aerobic and anaerobic conditions.

MATERIALS AND METHODS

For studies of intact erythrocytes, 0.2 ml of defibrinated sheep blood was added to 1.6 ml of media per vial. Other substrates tested include myoglobin (Nutritional Biochemical Corp., Cleveland, Ohio), cytochrome *c* from horse heart (Sigma Chemical Co., St. Louis, Mo.), bilirubin (Nutritional Biochemicals Corp.), hemin (iron protoporphyrin), iron hematoporphyrin, copper hematoporphyrin, and protoporphyrin. Each of these compounds was dissolved to give 0.75 μmole in a 1.8-ml portion of media. The protoporphyrin, hematoporphyrin, and their iron or copper complexes were prepared by standard procedures (16) and dissolved in minimal volumes of 0.05 to 0.2% NaOH so that the initial *p*H of the medium was 8.0 ± 0.1 after these porphyrin substrates were added. Human hemoglobin was prepared by the method of Quie and Wannamaker (14) and diluted to 25 mg (1.5 μmoles of heme) per 1.8 ml of media.

Todd-Hewitt broth (Difco Laboratories, Detroit, Mich.) was used for all experiments because some strains of streptococci did not grow as well on three alternative media. Pilot studies demonstrated that freshly prepared Todd-Hewitt medium produces small amounts of CO aerobically even when sterile. On exposure to light there was a sixfold increase in the rate of CO accumulation from Todd-Hewitt broth. Over a period of weeks in daylight, the Todd-Hewitt media lost much of its amber color, and the spontaneous rate of CO formation diminished. Since this medium contains a beef heart extract, the CO may be coming from hemoproteins. By storing open petri dishes with plain media and media plus added substrate inside larger closed dishes containing dry pellets of 0.5% palladium on ⅛-inch alumina pellets (Engelhard Industries Inc. Newark, N.J.) at room temperature for 24 hr, it was possible to deplete the media of dissolved CO prior to each study.

Each substrate was incubated with three strains of bacteria, obtained from throat cultures that produce alpha (green), beta (clear), or no hemolysis on aerobic 5% sheep blood agar plates. The Laboratory Division of the Center for Disease Control, Atlanta, Ga., identified the beta-hemolytic organism as a strain of *Bacillus cereus*, whereas both the alpha-hemolytic and the nonhemolytic bacteria were different strains of *Streptococcus mitis*. Testing on benzidine-erythrocyte-brain agar plates (9) characterized the alpha-hemolytic *S. mitis* as having the capacity to form H_2O_2 under aerobic conditions, whereas the nonhemolytic *S. mitis* did not make H_2O_2. Since the *B. cereus* produces catalase, its capacity to form H_2O_2 was not determined.

The bacterial inoculum was always standardized

as to quantity and growth phase by incubating a 6-ml subculture at 37 C for the 6 hr immediately preceding a study. After these subcultures were shaken, a 0.2-ml sample was added to 1.8 ml of plain medium for control samples or to 1.8 ml of medium containing substrate for experimental preparations. Shallow vials with different combinations of media, substrate, and bacteria were incubated in 100-ml glass syringes to collect any evolved CO. A film of mineral oil around the plunger and a metal cap on top of the syringe prevented gas leaks, as evidenced by failure of the syringe barrel to collapse on the supporting plunger.

The effect of an aerobic or anaerobic atmosphere was tested by flushing the syringe five times with either pure oxygen or nitrogen before capping it with the plunger retracted so that the liquid level was at the 100-ml mark. To reduce background CO in studies employing air, room air was drawn five times through a cartridge filled with palladium pellets before sealing the syringe. After incubating the cultures or controls in upright syringes for 18 hr at 37 C in the dark, the gas sample was analyzed by expelling the syringe through the 5-ml sample loop of a Perkin Elmer model 900 gas chromatograph. A thermal conductivity detector measured the H_2 and O_2 concentration, and a flame ionization detector responded to the CH_4 and CO content of the sample as described by Rodkey (15).

In our gas analysis, the CO attributed to bacteria was indistinguishable from that of a standard CO mixture by the following criteria: (i) the elution time from the gas chromatograph was identical; (ii) the flame ionization detector did not respond to samples from either source when the nickel catalyst was too cool to convert the CO to methane; and (iii) exposure to palladium pellets at room temperature removed the CO peak from either source. Although the analytic procedure can detect less than 0.5 nmole of CO in 100 ml ($\pm$0.1 ppm of CO) of air, only values exceeding 30 nmoles of CO are considered due to bacterial action because of variations in CO values found after sterile incubations.

The presence of bacterial growth and the absence of contaminants was always documented by streaking loops, from both the initial bacterial inoculum and from the culture after incubation, onto a blood agar plate. Inoculated vials always became turbid with flocculant growth.

The CO accumulated in the gas space of the syringe will be less than the total CO production if CO is retained in the liquid phase or if CO is consumed by bacteria. To test for loss of CO from the gas space, syringes were filled with a standard gas containing 54 nmoles of CO in 100 ml of air at standard temperature and pressure. When this gas was incubated for 18 hr with either sterile medium or medium plus alpha-, beta-, or nonhemolytic streptococci, 52 to 56 nmoles of CO was always recovered. When the medium contained 0.2 ml of sheep blood, then the difference between samples incubated overnight with CO-free room air and the standard gas containing CO was 45 nmoles. This presence of about 80% of the CO in the gas phase of the latter is the amount predicted from the Haldane relationship (4) and the CO affinity of sheep blood as reported by Sendroy and O'Neal (Fed. Proc. 14:137, 1955). Relatively more CO will be in the gas space in experiments with pure O_2 or no hemoprotein. Although proportionately more CO is bound to hemoglobin under anaerobic conditions, this was disregarded in the present calculations since the CO tensions in N_2 were always less than that required to saturate even 2% of the hemoglobin with CO. Also, there was no measure of whether the residual hemoprotein had retained the ability to bind CO. Therefore, the results on CO accumulation in the gas space have not been corrected for CO retention in the liquid phase.

Morphologic features of alpha and beta hemolysis were correlated with the chemical reactions under investigation by growing bacteria aerobically and anaerobically on 5% sheep blood agar plates with intact and lysed erythrocytes. To obtain plates with lysed erythrocytes, conventional 5% blood agar plates were alternately frozen and thawed three times. The visual evaluation of alpha- and beta-hemolytic zones was supplemented by microscopic inspection of the erythrocytes on frozen sections from blood agar plates. Red cell lysis and altered staining of intact red cells were delineated by applying azocarmine, Wright, Giemsa, and hematoxylin-eosin stains to these histologic sections.

RESULTS

In contrast to the nonhemolytic *S. mitis*, both the alpha-hemolytic *S. mitis* and the hemolytic *B. cereus* always liberated CO when incubated aerobically with iron porphyrins or defibrinated sheep blood (Table 1). None of these three bacterial strains showed a significant rise in CO accumulation above control levels when incubated with the same heme compounds in N_2, despite the presence of turbid bacterial growth. Neither the aerobic nor the anaerobic incubation of protoporphyrin, copper hematoporphyrin, or bilirubin with these three bacteria produced a significant rise in CO accumulation above control samples.

To ascertain whether the CO production observed with pure O_2 could also occur under atmospheric conditions, vials with the same combinations of sheep blood and bacteria were incubated with CO-free room air. Again, both hemolytic bacteria made CO aerobically, and nonhemolytic *S. mitis* did not (Table 1). However, the amount of CO evolved during the initial 18 hr was more with pure O_2 than with air.

After 18 hr of aerobic incubation the alpha-hemolytic *S. mitis* always liberated more CO in the presence of heme compounds than did the *B. cereus*. However, repeat measurements after 7 days of aerobic incubation with 25 mg of hemoglobin (equivalent to 1.5 μmoles of

TABLE 1. *Carbon monoxide production from heme compounds by hemolytic bacteria*

Bacteria	Gas phase	CO produced[a]									
		Hemoprotein added					Porphyrin added				
		None	Sheep blood	Hemoglobin	Myoglobin	Cytochrome *c*	Proto	Fe Proto	Fe Hemato	Cu Hemato	Bilirubin
Sterile	Air	5	8								
	O_2	10	6	28	8	12	17	7	11	6	3
	N_2	3	5	6	4	3	4	1	3	2	
Nonhemolytic *Streptococcus mitis*	Air	3	5								
	O_2	7	5	24	12	8	21	20	9	6	7
	N_2	1	5	8	3	7	1	2	2	1	
Alpha-hemolytic *S. mitis*	Air	8	*60*								
	O_2	10	*152*	*106*	*236*	*293*	20	*254*	*160*	11	8
	N_2	2	26	29	8	5	1	5	4	2	
Hemolytic *Bacillus cereus*	Air	6	*39*								
	O_2	9	*72*	*56*	*65*	*259*	20	*104*	*60*	9	5
	N_2	1	6	17	3	4	1	1	2	1	

[a] Each value is the mean for two or more experiments expressed as nanomoles of CO found in the gas phase after 18 hr of incubation at 37 C. Less than 30 nmoles of CO was found in all control samples.

heme) resulted in the accumulation of 1.5 μmoles of CO for the *B. cereus* and only 0.5 μmole of CO with the alpha-hemolytic *S. mitis*. Along with this difference in total CO evolved, the hemolytic *S. mitis* was often not viable on subcultures obtained on the seventh day, whereas *B. cereus* showed no loss of vigor. Under anaerobic conditions, none of the bacteria made CO in excess of sterile controls when the incubation period was extended to 7 days.

To verify that CO production is coupled with the destruction of heme compounds, absorption spectra were obtained on the supernatant fluid from centrifuged samples of the post-incubation mixture. All combinations that yielded significant CO accumulation had at least a proportionate decrease in the original absorption maxima between wavelengths 450 and 650 nm. In contrast, the absorption spectra remained relatively stable for most combinations that did not yield CO, such as anaerobic incubations with *B. cereus*, aerobic incubations with nonhemolytic *S. mitis*, and all incubations with protoporphyrin. Both strains of *S. mitis* showed changes in absorption maxima in disproportion to CO production. Thus, anaerobic incubations of the two strains of *S. mitis* with hemoglobin and cytochrome *c* yielded maxima at about 630 and 532 nm, respectively. (The 630 nm peak was also observed in aerobic incubations with hemoglobin.) In addition, the aerobically incubated alpha-hemolytic *S. mitis* showed loss of all specific absorption for hemoproteins, but formed only 0.33 μmole of CO per μmole of heme. Absorption spectra were not obtained on samples with copper hematoporphyrin, iron hematoporphyrin, or bilirubin.

Incidental to the CO measurements, the H_2, O_2, and CH_4 content of each gas sample was also determined. Neither H_2 nor CH_4 accumulated with any of these bacteria. *B. cereus* consumed appreciable amounts of O_2.

Other bacteria were also tested for CO production from sheep erythrocytes to see if this capacity is widely distributed. In every case nonhemolytic bacteria, for example *Streptococcus salivarius*, did not produce CO. In contrast, alpha-hemolytic bacteria such as *Streptococcus viridans* and beta-hemolytic bacteria (enterococci or Lancefield groups A and G streptococci) made CO when incubated aerobically with sheep blood. In general, hemolytic wild-type strains were better CO producers than hemolytic bacteria obtained from museum collections. Two attempts to demonstrate CO production by incubating sterile filtrates of the extracellular products from hemolytic streptococci with sheep blood or hemoglobin were unsuccessful.

The possibility that alpha hemolysis results

from microbial H_2O_2 formation has been considered by previous investigators (6, 7, 11), who noted that alpha-hemolytic pneumococcus and *S. viridans* are also H_2O_2 producers. Because our two strains of *S. mitis* differed with regard to H_2O_2 production and hemolysis, we also compared an H_2O_2-producing strain of *Streptococcus faecalis* with a peroxide-negative mutant, kindly supplied by Beulah Gray Holmes. In both cases the H_2O_2-producing organisms generated alpha hemolysis and formed CO from heme compounds, whereas the peroxide-negative bacteria did not demonstrate alpha hemolysis or CO formation. If, in alpha hemolysis, hemoglobin is converted into a green pigment without lysis of the red cell membrane, then the failure of this pigment to diffuse away from the colony would be explained. Microscopic inspection of frozen sections from 5% sheep blood agar plates supported this concept. Colonies of the alpha-hemolytic *S. mitis* and the H_2O_2-producing *S. faecalis* were surrounded by intact erythrocytes containing a pigment that was much darker staining than the hemoglobin of control erythrocytes when frozen sections were stained with azocarmine, Wright, Giemsa, hematoxylin, or eosin stains. A similar change in red cell staining was produced by adding a drop of H_2O_2 to the blood agar plate. In contrast, the nonhemolytic *S. mitis* and the H_2O_2-negative *S. faecalis* did not alter the staining characteristics of red cells. *B. cereus* lysed the cells. This would suggest that alpha-hemolytic bacteria convert hemoglobin to a green pigment by generating an extracellular substance that can pass through erythrocyte membranes. Although H_2O_2 can generate CO from hemoglobin (18), it is uncertain whether the catabolic sequence proceeds this far in alpha hemolysis, since both potential precursors (5) (verdoglobin and verdohemochrome) and by-products (22) (biliverdin) of CO production are green.

DISCUSSION

There are several similarities between previous reports of CO formation from heme by mammalian tissues and the present findings with bacteria. In each case CO production was observed only aerobically. Neither the bacteria nor mammalian tissues formed CO from free protoporphyrin, but both systems could utilize erythrocytes (2), hemoglobin (2), and hemin (2, 23). When Coburn et al. (2) injected ^{14}C-protoporphyrin into dogs and recovered ^{14}CO, they also obtained evidence that protoporphyrin is converted to heme before the formation of CO. Although Tenhunen et al. (23) reported almost no CO or bilirubin formation from myoglobin by the microsomal fraction of rat spleen cells, other studies have demonstrated in vivo (3) bilirubin and in vitro (19) CO formation from myoglobin, as was the case with the hemolytic bacteria in this study.

If mammalian tissues share the ability of bacteria to form CO from cytochrome *c*, then this substrate may partially account for reports (10) of CO formation in excess of hepatic bilirubin elimination. Schwartz et al. have shown that, in contrast to hemoglobin, the injection of isotopic cytochrome *c* in the dog is followed by excretion of labeled derivatives in the urine and not in the bile or feces (Fed. Proc. **27**:777, 1968). The formation of CO from cytochrome *c* by hemolytic bacteria raises the possibility that these microbes may derive a competitive advantage from a reaction that could provide them with iron or disrupt heme enzyme-dependent processes in phagocytic cells and other bacteria.

The stoichiometry between microbial heme destruction and CO production has not been defined by the present studies because competing reactions were not excluded and decreases in the absorption maxima could not be related quantitatively to degradation of substrates. After anaerobic incubation with alpha-hemolytic *S. mitis*, the initial absorption maxima were often eliminated or replaced by new peaks in the absence of CO accumulation. Further studies were not done to determine whether the heme compounds were also converted to products with an intact porphyrin ring. Jakob (8) has reported that pure cultures of bacteria can convert hemoglobin to protoporphyrin.

An excess of hemoglobin and hematin destruction over the production of CO and bilirubin has also been observed when isotopic heme compounds were given in vivo or in vitro to mammalian systems (2, 10, 12, 13, 21, 22). Pimstone et al. (13) found that macrophages formed CO and bilirubin in equimolar amounts. These authors suggested that in mammals H_2O_2 may promote the catabolism of hemoglobin by reactions that do not involve bilirubin production. Sjostrand (17) observed CO production on adding H_2O_2 to hemoglobin.

In the absence of isotopic labeling of the precursors or structural studies of the products, it is uncertain whether bacteria also derive CO from molecular oxygen plus the alpha methene carbon of the porphyrin ring. The mechanisms whereby microbes generate CO from heme compounds also remain to be elucidated. It has been shown that CO formation can accompany heme catabolism regardless of

whether the reaction is mediated by specific enzyme systems such as microsomal heme oxygenase (23) or nonspecific coupled oxidation systems such as ascorbic acid and H_2O_2 (18). Irrespective of whether microbial CO production is an intracellular or extracellular reaction, CO formation was not observed after bacteria ceased to be viable, or with sterile filtrates of extracellular fluid. This could reflect a requirement for a source of energy, for example reduced nicotinamide adenine dinucleotide phosphate by mammalian heme oxygenase (13, 23), or for a labile compound such as H_2O_2.

All six strains of bacteria that formed hemolytic zones on blood agar plates also produced CO from erythrocytes, and conversely the four strains that were nonhemolytic did not make CO. Despite this concordance, it appears that the formation of beta-hemolytic zones around colonies grown on blood agar plates does not depend on hemoglobin catabolism. Thus, prominent beta-hemolytic zones were formed under anaerobic conditions that prevented CO formation. Also, hemolysis but not CO formation was produced by sterile filtrates of the extracellular products from beta-hemolytic bacteria.

Clear zones resembling beta hemolysis can be produced by simply freeze-thawing a spot on a blood agar plate and then waiting a day for hemoglobin to diffuse away from the lysed cells. Although hemolytic *B. cereus* grown on aerobic hemoglobin agar plates gradually produced diffuse color changes, the discrete zones observed on plates with sheep erythrocytes were absent. This supports Beswick's finding that the clear zones around colonies of beta-hemolytic bacteria result primarily from erythrocyte membrane lysis permitting hemoglobin to diffuse away (1). Although Beswick did not detect any decolorization of hemoglobin by beta-hemolytic bacteria, it is apparent that this can occur at a slow rate, as evidenced by the data on CO formation and by color loss of hemoglobin agar plates after several days of aerobic but not anaerobic incubation. Since *B. cereus* forms catalase it is doubtful that this organism depends on H_2O_2 either for CO formation or for the gradual decolorization of hemoglobin under aerobic conditions.

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